

# PU.1 Regulates Expression of the Interleukin-7 Receptor in Lymphoid Progenitors

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## Summary

Development of the lymphoid system is dependent on the Ets family transcription factor PU.1. We demonstrate that *PU.1*<sup>-/-</sup> hematopoietic progenitors fail to express *IL-7R $\alpha$*  transcripts. Promoter and chromatin crosslinking analyses suggest that PU.1 directly regulates transcription of the *IL-7R $\alpha$*  gene. Retroviral transduction of *IL-7R $\alpha$*  into *PU.1*<sup>-/-</sup> progenitors restores IL-7-dependent proliferation and induces, at low frequency, the generation of pro-B cells undergoing an apparently normal program of differentiation. Although the related factor Spi-B can substitute for PU.1 in early B cell development, it is not required. These results demonstrate that PU.1 functions to regulate early B cell development in part by controlling the expression of the *IL-7R $\alpha$*  gene.

## Introduction

Various transcription factors have been shown by loss- and gain-of-function experiments to be required for early B cell development, including *PU.1*, *Ikaros*, *E2A*, *EBF*, and *Pax5* (reviewed in O’Riordan and Grosschedl, 2000). Phenotypic analyses of mice with targeted null alleles of genes encoding these factors have been used to order their developmental functions in the B lineage. *PU.1* and *Ikaros* appear to act upstream of *E2A*, *EBF*, and *Pax5* at the level of multipotential progenitors (Scott et al., 1994; McKercher et al., 1996; Georgopoulos et al., 1994; Wang et al., 1996). In contrast, *E2A*, *EBF*, and *Pax5* function in B lineage progenitors (Zhuang et al., 1994; Bain et al., 1994; Lin and Grosschedl, 1995; Nutt et al., 1997, 1999). *E2A*, *EBF*, and *Pax5* directly control transcription of early B cell genes (*mb-1*, *B29*, *VpreB*,  $\lambda 5$ , *RAG-1*, and *RAG-2*) as well as their own hierarchical expression (reviewed in O’Riordan and Grosschedl, 2000). *E2A* appears to directly regulate transcription of the *EBF* gene (Kee and Murre, 1998) and EBF in turn is implicated in activating expression of the *Pax5* gene (O’Riordan and Grosschedl, 1999). PU.1 and *Ikaros* do not appear to regulate each others’ expression (Scott et al., 1997; Nichogiannopoulou et al., 1999) and may act independently of one another. PU.1 has been implicated in regulating the activity of the *IgH* intronic en-

hancer (Nikolajczyk et al., 1999), whereas *Ikaros* proteins have been suggested to repress T lineage genes in B cells via association with centromeric heterochromatin (Brown et al., 1997). However, these proposed molecular functions do not readily account for the blocks to B cell development observed in *PU.1* and *Ikaros* knock-out mice.

Mice homozygous for a null mutation in the *PU.1* gene die during fetal development by 18.5 days post coitum (d.p.c.) and lack B, T, and myeloid progenitors. (Scott et al., 1994, 1997). *PU.1*<sup>-/-</sup> fetal liver contains reduced numbers of multipotential lymphoid-myeloid progenitors (AA4.1<sup>+</sup>, Lin<sup>-</sup>). Furthermore, these mutant progenitors fail to proliferate and differentiate into pro-B cells in response to stromal contact and Interleukin-7 (IL-7) (Scott et al., 1997). There are two mutually nonexclusive explanations for the defects to lymphoid development in *PU.1*<sup>-/-</sup> fetuses. First, PU.1 might regulate lymphoid differentiation by functioning at the top of a genetic hierarchy to control expression of downstream regulators such as *E2A*, *EBF*, and *Pax5*. Alternatively, PU.1 may regulate genes necessary for survival and proliferation of lymphoid progenitors.

Survival and proliferation of lymphoid progenitors in fetal liver and bone marrow are dependent on cytokine signaling. Several cytokines have been demonstrated to be important for developing B and T cells, including stem cell factor (SCF), Flt-3 ligand, and IL-7 (reviewed in Di Santo and Rodewald, 1998; Baird et al., 1999). IL-7 is produced primarily by stromal cells and is essential for the proliferation and differentiation of T and B cell progenitors in vivo (Goodwin et al., 1990; von Freeden-Jeffry et al., 1995). The receptor for IL-7 is heterodimeric, consisting of an  $\alpha$  chain (IL-7R $\alpha$ ) (Goodwin et al., 1990) and a common  $\gamma$  chain ( $\gamma$ c) (Noguchi et al., 1993; Kondo et al., 1994b). The  $\gamma$ c subunit is shared with the IL-2, IL-4, IL-9, and IL-15 receptors (reviewed in Sugamura et al., 1995), while the IL-7R $\alpha$  chain has recently been found to be shared by the thymic stromal lymphopoietin (TSLP) receptor (Pandey et al., 2000; Park et al., 2000). IL-7R $\alpha$  is primarily expressed in fetal liver and bone marrow common lymphoid progenitors (CLPs), T lineage cells, and developing B cells (Mebius et al., 2001; Kondo et al., 1997b; Sudo et al., 1993), while  $\gamma$ c is more broadly expressed in various hematopoietic lineages (He et al., 1995; Kondo et al., 1994a). Targeted mutations of either the *IL-7R $\alpha$*  (Peschon et al., 1994; Maki et al., 1996) or  $\gamma$ c (DiSanto et al., 1995; Cao et al., 1995) genes result in a profound early block to B and T cell development. Ectopic expression of bcl-2 partially restores  $\alpha\beta$ <sup>+</sup> T cell but not B cell development (Akashi et al., 1997; Maraskovsky et al., 1997; Kondo et al., 1997a). This result suggests that IL-7 signaling is required not only for survival and proliferation of B lineage progenitors but also for their differentiation.

*PU.1*<sup>-/-</sup> hematopoietic progenitors have been shown to be impaired in the expression of several myeloid cytokine receptor genes including *GM-CSFR $\alpha$* , *G-CSFR*, and *c-fms* (DeKoter et al., 1998). Therefore we examined the possibility that the defect to B and T cell development

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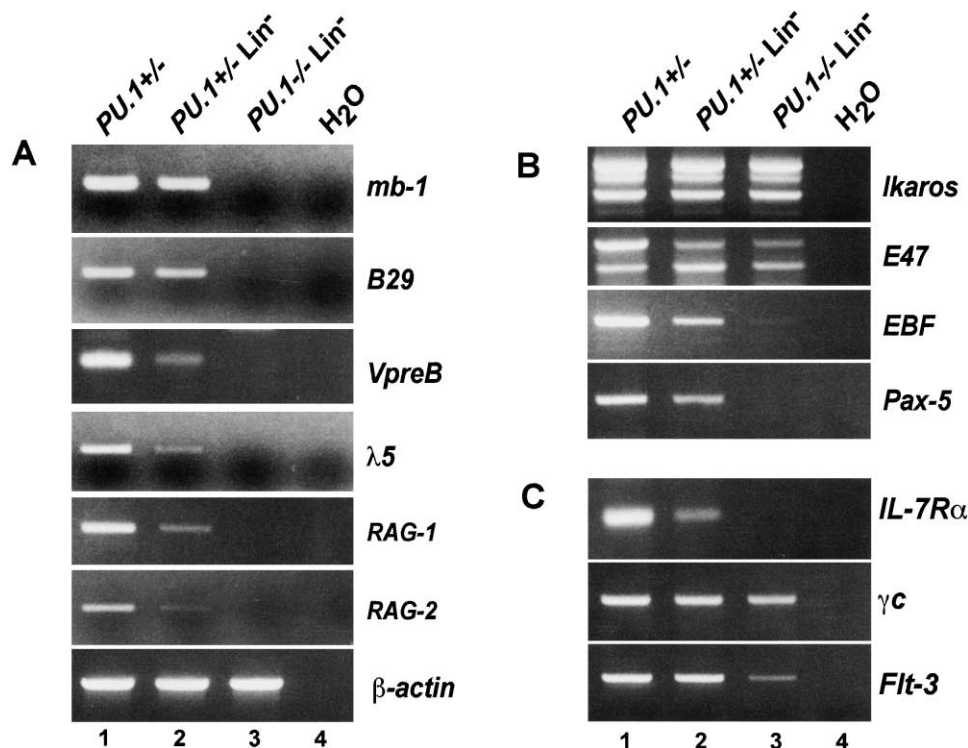


Figure 1. Analysis of Early B Lineage Gene Expression in *PU.1*<sup>+/+</sup> and *PU.1*<sup>-/-</sup> Hematopoietic Progenitors

Total RNA was prepared from whole 14.5 d.p.c. fetal liver (*PU.1*<sup>+/+</sup>) or from lineage-depleted (Lin<sup>-</sup>) fetal liver progenitors from *PU.1*<sup>+/+</sup> and *PU.1*<sup>-/-</sup> fetuses. The indicated transcripts were analyzed by RT-PCR after normalization with  $\beta$ -actin.

in *PU.1*<sup>-/-</sup> progenitors might be partly due to failed expression of genes encoding lymphoid cytokine receptors. We show that *PU.1*<sup>-/-</sup> progenitors are defective in expression of the *IL-7R $\alpha$*  gene. Retroviral transduction of *IL-7R $\alpha$*  into *PU.1*<sup>-/-</sup> progenitors restores their IL-7-dependent proliferation and at low frequency their differentiation into pro-B cells that express the *mb-1*, *B29*, *VpreB*,  $\lambda$ 5, *RAG-1*, and *RAG-2* genes and undergo *IgH* D-J rearrangement. IL-7R-rescued differentiation does not require the related Ets factor Spi-B. These results demonstrate that PU.1 regulates early B cell development in part by controlling the expression of the *IL-7R $\alpha$*  gene.

## Results

### *PU.1*<sup>-/-</sup> Hematopoietic Progenitors Fail to Express *IL-7R $\alpha$* Transcripts

To define the nature of the block to B cell development in *PU.1*<sup>-/-</sup> fetuses, we prepared RNA from multipotential progenitors (Lin<sup>-</sup>) of 14.5 d.p.c. *PU.1*<sup>+/+</sup> or *PU.1*<sup>-/-</sup> fetal liver using a lineage depletion protocol (DeKoter and Singh, 2000). RT-PCR analysis showed that genes encoding B cell receptor-associated proteins (*mb-1*, *B29*, *VpreB*, and  $\lambda$ 5) as well as the recombinase (*RAG-1* and *RAG-2*) were not expressed in *PU.1*<sup>-/-</sup> progenitors (Figure 1A). Genes encoding the transcription factors Ikaros and E47 were expressed in *PU.1*<sup>-/-</sup> progenitors at levels comparable to *PU.1*<sup>+/+</sup> controls whereas *EBF* transcripts were barely detectable and *Pax5* transcripts were absent (Figure 1B). The expression of *EBF* and *Pax5* genes

is restricted to B cells within the hematopoietic system (Hagman et al., 1993; Adams et al., 1992), whereas *Ikaros* and *E2A* genes are expressed in multiple hematopoietic lineages (Nichogiannopoulou et al., 1999; Murre et al., 1989). These results extend our previous analysis (Scott et al., 1997) and suggest that B lymphoid progenitors are absent in *PU.1*<sup>-/-</sup> fetal liver.

To determine whether *PU.1*<sup>-/-</sup> hematopoietic progenitors express genes encoding cytokine receptors involved in lymphoid development, RT-PCR analysis was performed for IL-7R subunits (*IL-7R $\alpha$* ,  $\gamma$ C) and *Flt3*.  $\gamma$ C and *Flt3* were expressed in *PU.1*<sup>-/-</sup> progenitors (Figure 1C). The lower levels of *Flt3* transcripts are likely due to a reduction in *Flt3*<sup>+</sup> progenitors in *PU.1*<sup>-/-</sup> fetal liver (K. Medina and H.S., unpublished data). Strikingly, expression of *IL-7R $\alpha$*  transcripts was undetectable in *PU.1*<sup>-/-</sup> progenitors even after two rounds of PCR amplification (Figure 1C and data not shown). This result could be due to the absence of lymphoid progenitors in *PU.1*<sup>-/-</sup> fetal liver. Alternatively, PU.1 could directly regulate expression of the *IL-7R $\alpha$*  gene and therefore the absence of lymphoid progenitors in *PU.1*<sup>-/-</sup> fetal liver would be due in part to the failed expression of the IL-7R.

### PU.1 Directly Regulates Transcription of the *IL-7R $\alpha$* Gene

To explore if PU.1 directly regulates transcription of the *IL-7R $\alpha$*  gene, the DNA sequence spanning the transcriptional start site (-2495 to +946, Pleiman et al., 1991) was examined for potential PU.1 binding sites using a

position-weight matrix algorithm (Ludwig et al., 2000). Two putative sites were identified, located at -914 and +763 relative to the transcription start site (Figure 2A). Comparison with the human *IL-7R $\alpha$*  promoter sequence (Puel et al., 1998) revealed a conserved region around site 2 but not site 1. We note that site 2 is located at positions -182 and -180 relative to the translational start sites in the murine and human genes, respectively. Gel shift analysis with oligonucleotide probes containing these sequences revealed that PU.1 bound the *IL-7R $\alpha$*  gene site 2 with a higher affinity than site 1 (Figure 2B). The *IL-7R $\alpha$*  site 2 has a comparable affinity for PU.1 as the  $\lambda$ B site in an *Ig $\lambda$*  gene enhancer (Figure 2B). We note that the *IL-7R $\alpha$*  site 2, and not a mutant version (see below), is specifically bound by endogenous PU.1 protein in nuclear extracts from 38B9 pro-B cells (data not shown). Therefore the murine *IL-7R $\alpha$*  gene contains a high-affinity PU.1 binding site that lies between the transcriptional and translational start sites.

Several murine *IL-7R $\alpha$*  promoter constructs were tested for activity by transient transfection in B lineage cell lines that express both PU.1 and the IL-7R. However, we detected very low activity of the promoter fragments using this approach, preventing functional analysis of the PU.1 binding sites in their native context. To test whether site 2 could promote PU.1-dependent activation in vivo, three tandem repeats of the intact or mutated site were cloned into a luciferase reporter construct upstream of a minimal SV40 promoter. Transfection into 38B9 pro-B cells, which express both PU.1 and IL-7R, showed that the intact but not the mutated PU.1 binding sites stimulated transcription by 10-fold (Figure 2C). In NIH-3T3 cells, which do not express PU.1, ectopically expressed PU.1 stimulated transcription from the *IL-7R $\alpha$*  site 2 reporter construct (data not shown).

To test whether the *IL-7R $\alpha$*  gene binding site 2 is occupied by PU.1 protein in vivo, chromatin immunoprecipitation (ChIP) experiments were performed. Affinity-purified anti-PU.1 antibody was used to immunoprecipitate chromatin prepared from IL-7-dependent, wild-type pro-B cells. PCR analysis was performed on DNA prepared from immunoprecipitated chromatin using primers spanning *IL-7R $\alpha$*  gene PU.1 sites 1, 2, or exon 5. DNA fragments containing PU.1 site 2 were enriched by an average of 12-fold ( $n = 3$ ) over fragments containing site 1 or exon 5 sequences (Figure 3A). Therefore PU.1 associates with the *IL-7R $\alpha$*  gene in pro-B cells. To determine if PU.1 is bound to the *IL-7R $\alpha$*  gene in other cellular contexts, we carried out ChIP analyses in WEHI-231 B cells and J774.1 macrophages. WEHI-231 cells do not express the *IL-7R $\alpha$*  gene whereas low levels of *IL-7R $\alpha$*  transcripts are detectable in J774.1 cells (data not shown). PU.1 was found to be crosslinked to the *IL-7R $\alpha$*  gene site 2 in J774.1 cells but not in WEHI-231 cells (Figure 3B). As additional controls for ChIP, we examined the binding of PU.1 to the *Ig $\kappa$*  3' enhancer as well as the *c-fms* promoter in WEHI-231 and J774.1 cells. PU.1 crosslinking to these regulatory elements was cell type specific and correlated with the activity states of these loci (Figure 3B and data not shown). In conclusion, we have identified a functional high-affinity PU.1 binding site in the murine *IL-7R $\alpha$*  gene that is occupied by PU.1 in pro-B cells. These results, along with the absence of

*IL-7R $\alpha$*  transcripts in *PU.1*<sup>-/-</sup> progenitors, suggest that PU.1 directly activates transcription of the *IL-7R $\alpha$*  gene.

#### Retroviral Transduction of *PU.1*<sup>-/-</sup> Progenitors with *IL-7R $\alpha$* Restores IL-7-Dependent Proliferation and Differentiation into Pro-B Cells

To test if restoration of IL-7R $\alpha$  expression might be sufficient to rescue IL-7-dependent proliferation of *PU.1*<sup>-/-</sup> progenitors, we utilized murine stem cell virus (MSCV) based vectors (Hawley et al., 1994) that contained *PU.1* or *IL-7R $\alpha$*  cDNAs (Figure 4A). The control and PU.1 retroviral vectors contain a GFP gene whose expression is made possible by an internal ribosome entry site (IRES) (Pear et al., 1998). The *IL-7R $\alpha$*  vector was constructed without an IRES-GFP cassette to facilitate secondary transductions using GFP based vectors. Lin<sup>-</sup> *PU.1*<sup>+/-</sup> or *PU.1*<sup>-/-</sup> hematopoietic progenitors were infected by coculture with retroviral packaging cells, then plated on S17 cells with IL-7 (DeKoter and Singh, 2000). *PU.1*<sup>+/-</sup> progenitors infected with control (MIGR1) virus proliferated and differentiated after 10–14 days into CD19<sup>+</sup> pro-B cells ( $91.9 \pm 2.6$ ,  $n = 8$ ) (Figure 4B, left panel), and CD19<sup>-</sup> Mac-1<sup>+</sup> macrophages ( $3.9 \pm 1.6\%$ ,  $n = 8$ ). *PU.1*<sup>-/-</sup> progenitors infected with MIGR1 virus failed to proliferate and died during the initial coculture ( $n = 8$ ). Consistent with earlier analysis (Scott et al., 1997), *PU.1*<sup>-/-</sup> progenitors could not be propagated on stroma with IL-7. In contrast, infection with MIG-PU.1 retrovirus restored the ability of *PU.1*<sup>-/-</sup> progenitors to proliferate in response to IL-7 and stromal contact. *PU.1*<sup>-/-</sup> progenitors transduced with *PU.1* cDNA generated CD19<sup>+</sup> pro-B cells ( $49.1 \pm 4.9\%$ ,  $n = 8$ ) (Figure 4B, middle panel), as well as CD19<sup>-</sup> Mac-1<sup>+</sup> macrophages ( $29.0 \pm 4.5\%$ ,  $n = 8$ ).

Transduction with *IL-7R $\alpha$*  cDNA restored the ability of *PU.1*<sup>-/-</sup> progenitors to proliferate in response to IL-7 and stromal contact. Interestingly, a small fraction ( $1.1 \pm 0.9\%$ ,  $n = 10$ ) of the transduced *PU.1*<sup>-/-</sup> cells expressed CD19 (Figure 4B, right panel), suggesting that pro-B cells are generated. However, most cells in these initial cultures did not express CD19 and morphologically resembled myeloid progenitors (data not shown). The apparent lower frequency of CD19<sup>+</sup> cells generated by transduction of *PU.1*<sup>-/-</sup> progenitors with *IL-7R $\alpha$*  compared to *PU.1* was confirmed by clonal analysis in which *PU.1*<sup>-/-</sup> progenitors were plated in methylcellulose cultures containing S17 stromal cells and IL-7 immediately following retroviral infection. Similar frequencies of IL-7-dependent colonies were generated from *PU.1*<sup>-/-</sup> progenitors infected with either *PU.1* or *IL-7R $\alpha$*  cDNAs (data not shown). However, whereas 27% (15/56) of colonies obtained by transduction of *PU.1* cDNA consisted of CD19<sup>+</sup> cells, the frequency of CD19<sup>+</sup> colonies generated with *IL-7R $\alpha$*  cDNA was below detection (0/46). These results suggest that PU.1 is more efficient than the IL-7R at restoring the differentiation of *PU.1*<sup>-/-</sup> progenitors into pro-B cells.

To analyze *PU.1* or *IL-7R $\alpha$* -transduced *PU.1*<sup>-/-</sup> progenitors in greater detail, cell lines were established by passage on S17 stromal cells with IL-7 (Rolink et al., 1991, 1993). Under these conditions, macrophages are lost after a few passages since the cells adhere to the stroma and do not substantially proliferate. After two

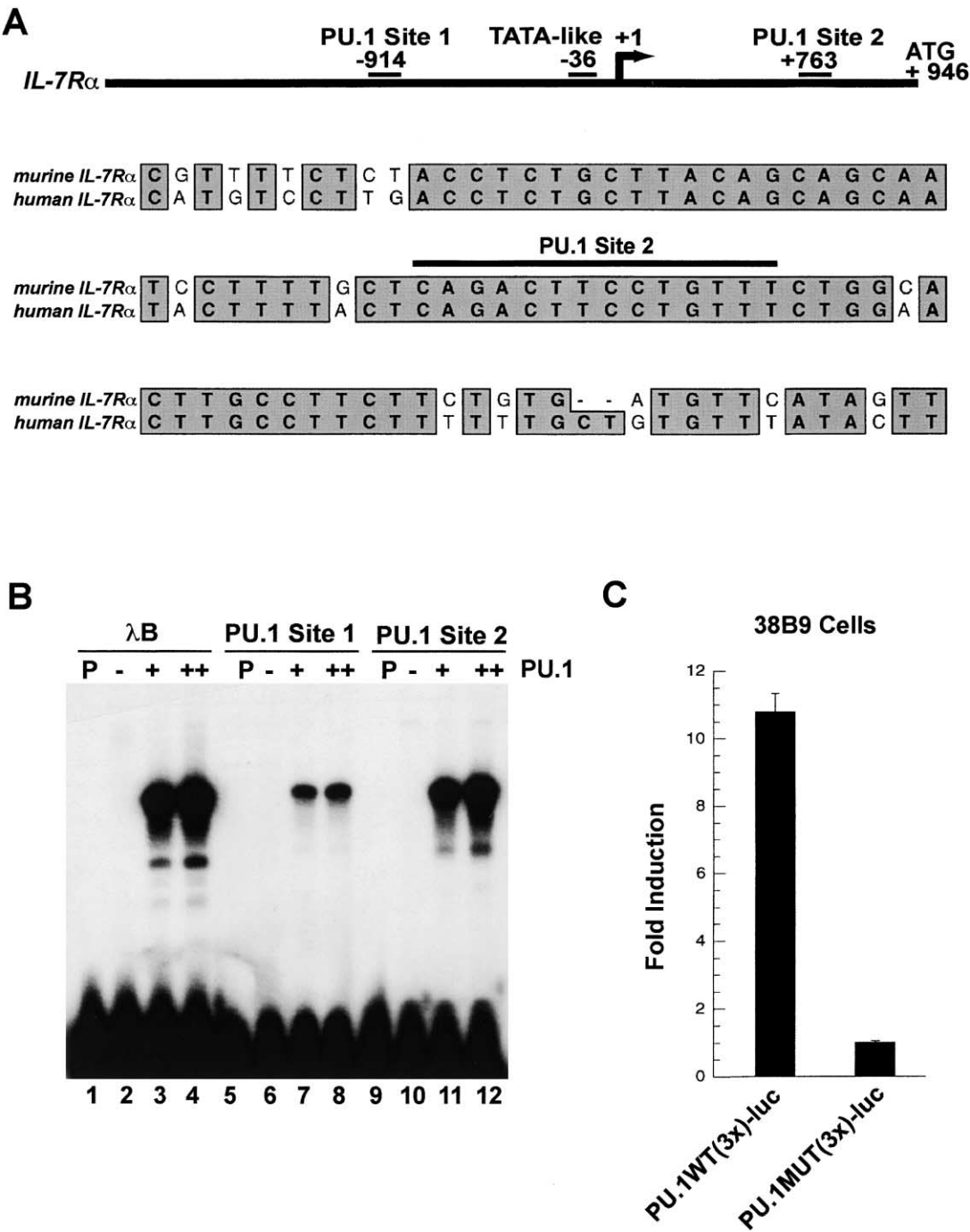


Figure 2. Analysis of PU.1 Binding Sites in the *IL-7R $\alpha$*  Gene

(A) Schematic structure of the murine *IL-7R $\alpha$*  gene promoter and downstream region indicating the PU.1 binding sites that were identified using a position weight matrix algorithm. Sequence alignment of a conserved region spanning PU.1 binding site 2 in the murine and human *IL-7R $\alpha$*  genes is shown below the schematic.

(B) Gel shift assay with PU.1 binding sites in the *IL-7R $\alpha$*  gene. A control high-affinity PU.1-binding site ( $\lambda$ B), PU.1 site 1, or PU.1 site 2 oligonucleotides were incubated with PU.1 protein generated by in vitro translation. Lanes 1, 5, and 9 contain free probes (P) whereas lanes 2, 6, and 10 represent incubations with control translation lysates (-). PU.1 translation lysates were used at two different concentrations, 2  $\mu$ l (+) and 4  $\mu$ l (++).

(C) PU.1 can activate transcription from the downstream *IL-7R $\alpha$*  binding site. pGL3-based reporter constructs containing three tandem copies of the wild-type or mutant PU.1 binding site 2 were transiently transfected into 38B9 pro-B cells. After normalization with a reference construct, the luciferase activities of the two reporters are represented in relation to the control pGL3 reporter.

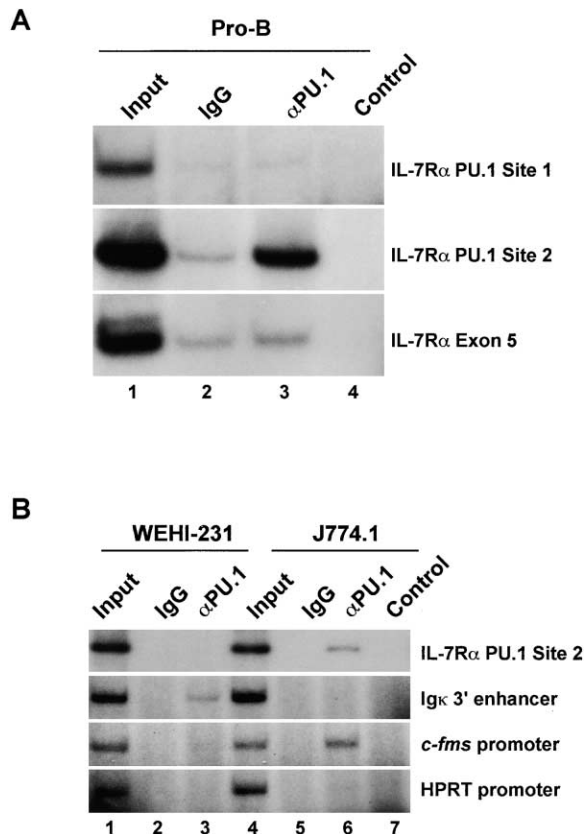


Figure 3. Chromatin Immunoprecipitation Analysis of PU.1 Binding to the *IL-7Rα* Gene

(A) Formaldehyde-crosslinked chromatin from wild-type fetal liver-derived pro-B cells was immunoprecipitated using control rabbit IgG or affinity purified anti-PU.1 ( $\alpha$ PU.1) antibodies as described in the Experimental Procedures. PCR was performed on input and immunoprecipitated DNA using primer pairs spanning PU.1 binding site 1, binding site 2, and exon 5 in the *IL-7Rα* gene. PCR reactions included  $\alpha$ - $^{32}$ P dCTP and the products were visualized by autoradiography. The control lane represents a PCR reaction conducted in the absence of template DNA.

(B) In vivo crosslinking of PU.1 in WEHI-231 B cells and J774.1 macrophages. Formaldehyde-crosslinked chromatin was analyzed as described in (A) using primer pairs spanning PU.1 binding sites in the *IL-7Rα* gene, *Igκ* 3' enhancer, and *c-fms* promoter. The HPRT gene promoter was used as a negative control.

passages, *PU.1*<sup>+/-</sup> and *PU.1*-rescued *PU.1*<sup>-/-</sup> cell lines contained exclusively CD19<sup>+</sup> pro-B cells (Figure 5A, top panel). Surprisingly, after two passages, cultures of *IL-7Rα*-transduced *PU.1*<sup>-/-</sup> cells contained a majority (61  $\pm$  4%, *n* = 3) of CD19<sup>+</sup> cells, suggesting that these cells proliferate more rapidly than the CD19<sup>-</sup> cells in the population (Figure 5A, top panel). Both *PU.1* and *IL-7Rα*-transduced *PU.1*<sup>-/-</sup> progenitors expressed *IL-7Rα* on their surface, albeit at lower levels than their control counterparts (Figure 5A, bottom panel). RT-PCR analysis indicated that the *IL-7Rα*-transduced cell lines express various B lineage genes including *mb-1*, *B29*, *VpreB*,  $\lambda$ 5, *RAG-1*, *RAG-2*, *EBF*, and *Pax5* (Figure 5B). PCR analysis demonstrated that these cell lines were also undergoing *DH-JH* rearrangement at their immunoglobulin heavy chain loci (Figure 5C). Since the *IL-7Rα*-

transduced cell lines comprise of both CD19<sup>+</sup> and CD19<sup>-</sup> cells, we reasoned that it is the former that likely express B lineage genes and undergo a normal program of B cell differentiation. To test this possibility, four CD19<sup>+</sup> as well as nine CD19<sup>-</sup> clones were established from early-passage cell lines and characterized in detail. Clones were analyzed by RT-PCR for expression of the B lineage genes *mb-1*, *B29*, *VpreB*,  $\lambda$ 5, *RAG-1*, and *RAG-2* and the myeloid genes *G-CSFR* and *FcγRIII*. As expected, the CD19<sup>+</sup> clones expressed various B cell-specific genes but did not express the myeloid genes *G-CSFR* or *FcγRIII* (Figure 6B). In contrast, CD19<sup>-</sup> clones expressed the myeloid but not the B lineage genes. It should be noted that expression of the *G-CSFR* gene is not absolutely dependent on PU.1 (DeKoter et al., 1998). Additionally, CD19<sup>+</sup> but not CD19<sup>-</sup> clones expressed the combination of CD24, CD43, *VpreB*, and *c-kit* on their surface and exhibited *DH-JH* rearrangements at their *IgH* loci (data not shown). Therefore the CD19<sup>+</sup> clones are pro-B cells, whereas the CD19<sup>-</sup> clones are likely myeloid progenitors that can proliferate in IL-7 as a consequence of ectopic expression of IL-7R.

#### Spi-B Can Rescue Differentiation of *PU.1*<sup>-/-</sup> Progenitors into Pro-B Cells and Macrophages

Spi-B and Spi-C (Prf) are Ets-family transcription factors that share a higher degree of identity with PU.1 than with any other Ets family member and appear to have DNA binding specificities indistinguishable from that of PU.1 (Ray et al., 1992; Ray-Gallet et al., 1995; Rao et al., 1999; Hashimoto et al., 1999; Bemark et al., 1999). Unlike PU.1, Spi-B and Spi-C are expressed exclusively in the lymphoid lineages (Su et al., 1996; Hashimoto et al., 1999; Bemark et al., 1999). *Spi-B* transcripts were detectable by RT-PCR in pro-B cell lines and clones derived from *IL-7Rα*-transduced *PU.1*<sup>-/-</sup> progenitors (Figure 7B and data not shown). In contrast, *Spi-C* transcripts were not detectable in the rescued pro-B cell lines (Figure 7B, lanes 3 and 4). The latter result is not surprising since Spi-C appears to be expressed only in mature B cells (Figure 7B, lane 1; Bemark et al., 1999). These results raised the possibility that Spi-B might promote differentiation of *IL-7Rα*-transduced progenitors into pro-B cells in the absence of PU.1. To test this hypothesis, *PU.1*<sup>-/-</sup> progenitors were transduced with *Spi-B* cDNA. *Spi-B* rescued the ability of *PU.1*<sup>-/-</sup> progenitors to proliferate in response to stromal contact and IL-7 and restored their ability to differentiate into CD19<sup>+</sup> pro-B cells (35.3  $\pm$  1.5%, *n* = 2) as well as Mac-1<sup>+</sup> macrophages (36.9  $\pm$  1.2%, *n* = 2) (Figure 7A). The proportions of the two cell types were similar to those obtained using *PU.1* cDNA (compare Figure 7A to Figure 4B). Furthermore, IL-7-dependent pro-B cell lines established from *Spi-B*-transduced *PU.1*<sup>-/-</sup> progenitors expressed the identical complement of B lineage genes and markers as described for their PU.1 counterparts (data not shown). Finally, as we have reported for PU.1, Spi-B-rescued macrophages expressed higher levels of GFP than their pro-B cell counterparts. This suggests, as is the case for PU.1, that higher levels of Spi-B are required for promoting macrophage than B cell development (DeKoter and Singh, 2000). In conclusion, Spi-B can functionally substitute for PU.1 in promoting differ-

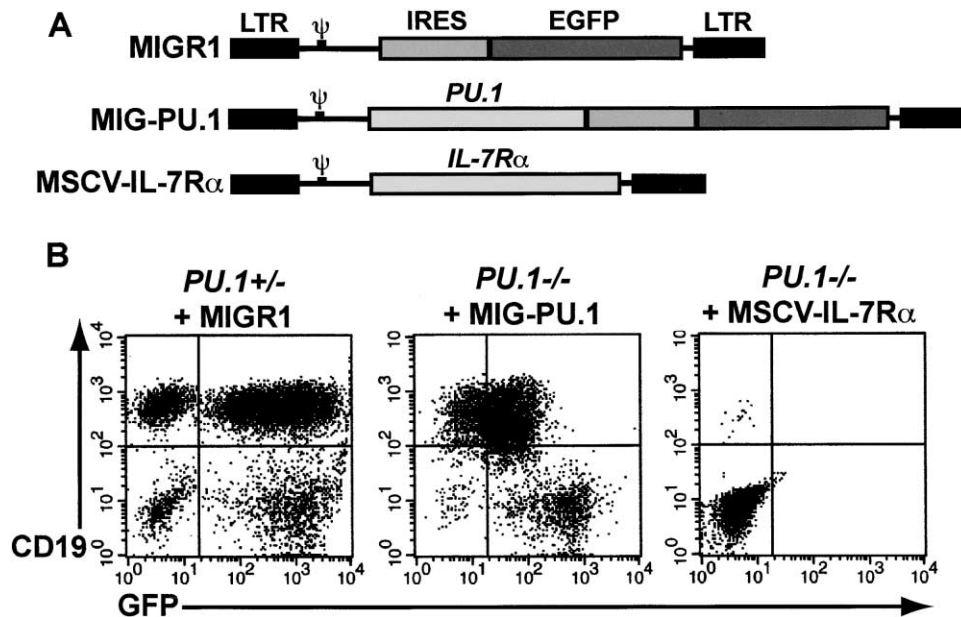


Figure 4. Transduction with an *IL-7R $\alpha$*  Retroviral Vector Restores IL-7-Dependent Proliferation of *PU.1*<sup>-/-</sup> Progenitors

(A) Schematic structures of MSCV-based retroviral constructs. LTR, long terminal repeat; IRES, internal ribosomal entry site; and EGFP, enhanced green fluorescent protein.

(B) Flow cytometric analysis of cultures of *PU.1*<sup>+/-</sup> or *PU.1*<sup>-/-</sup> progenitors after retroviral transduction. Lin<sup>-</sup> progenitors were transduced with MIGR1, MIG-PU.1, or MSCV-IL-7R $\alpha$  retroviral vectors by coculture with retroviral packaging cell lines. Transduced progenitor cells were expanded for 10–14 days on S17 stromal cells with Interleukin-7 (IL-7). Cells from these cultures were analyzed by flow cytometry for expression of CD19 and GFP.

entiation of *PU.1*<sup>-/-</sup> progenitors into pro-B cells and macrophages.

#### IL-7R-Dependent B Cell Development of *PU.1*<sup>-/-</sup> Progenitors Does Not Require Spi-B

To directly test the requirement of Spi-B in the IL-7R rescue of early B cell differentiation, we infected *PU.1*<sup>-/-</sup> *Spi-B*<sup>-/-</sup> (Garrett-Sinha et al., 1999) Lin<sup>-</sup> progenitors with the *IL-7R $\alpha$*  retroviral vector. Transduction with *IL-7R $\alpha$*  rescued stromal cell and IL-7-dependent proliferation of *PU.1*<sup>-/-</sup> *Spi-B*<sup>-/-</sup> progenitors. After 10–14 days, a small fraction of these cells expressed CD19 ( $1.0 \pm 0.7\%$ ,  $n = 3$ ). As was the case with *IL-7R $\alpha$*  transduction of *PU.1*<sup>-/-</sup> progenitors, after two passages the *PU.1*<sup>-/-</sup> *Spi-B*<sup>-/-</sup> cell lines contained a high proportion ( $59 \pm 6\%$ ,  $n = 2$ ) of CD19<sup>+</sup> cells. *PU.1*<sup>-/-</sup> *Spi-B*<sup>-/-</sup> CD19<sup>+</sup> cells were pro-B cells by all criteria described above including expression of B cell-specific transcripts, *DH-JH* rearrangement, and cell surface expression of B lineage markers (Figure 7B and data not shown). The rescued cells did not express Spi-C transcripts (Figure 7B, lanes 5 and 6). Therefore neither Spi-B nor Spi-C is required for the differentiation of *IL-7R $\alpha$* -transduced, *PU.1*<sup>-/-</sup> progenitors into CD19<sup>+</sup> pro-B cells.

#### Discussion

In this study, we have investigated the underlying basis of the profound lymphoid deficiency caused by mutation of the *PU.1* gene. We demonstrate that PU.1 is required for the expression of the IL-7R. Since *PU.1*<sup>-/-</sup> hemo-

poietic progenitors also lack early B lineage gene transcripts, we suggest that loss of PU.1 results in impaired survival and/or proliferation of lymphoid progenitors. Consistent with this finding, PU.1 is expressed in purified bone marrow common lymphoid progenitors (CLPs) (Akashi et al., 2000), pro-B cells (DeKoter and Singh, 2000), and pro-T cells (Anderson et al., 1999). A requirement for PU.1 in the expression of the IL-7R in lymphoid progenitors would explain several aspects of the *PU.1*<sup>-/-</sup> phenotype. IL-7R signaling is critical for B cell development, is less stringently required for  $\alpha\beta$ <sup>+</sup> T cell development, and is dispensable for NK cell development (Peschon et al., 1994; Maki et al., 1996). In accord with these findings, mutation of the *PU.1* gene results in a profound block to B cell development (Scott et al., 1994, 1997; McKercher et al., 1996; Colucci et al., 2001). In contrast, loss of *PU.1* function impairs or delays, but does not completely block,  $\alpha\beta$ <sup>+</sup> T cell development (Spain et al., 1999; McKercher et al., 1996). Finally, *PU.1*<sup>-/-</sup> progenitors are capable of differentiating into NK cells after transplantation into recipient mice, although the NK cells that develop in these hosts are defective in several ways, including in the expression of *IL-7R $\alpha$*  (Colucci et al., 2001). Therefore failed expression of IL-7R in hematopoietic progenitors is likely a contributing cause of the developmental defects of the lymphoid system in *PU.1*<sup>-/-</sup> fetuses.

After transduction of *PU.1*<sup>-/-</sup> hematopoietic progenitors with *IL-7R $\alpha$*  cDNA, differentiation into pro-B cells is observed, albeit at low frequency. These rare *PU.1*<sup>-/-</sup> pro-B cells, after expansion, exhibit an apparently nor-

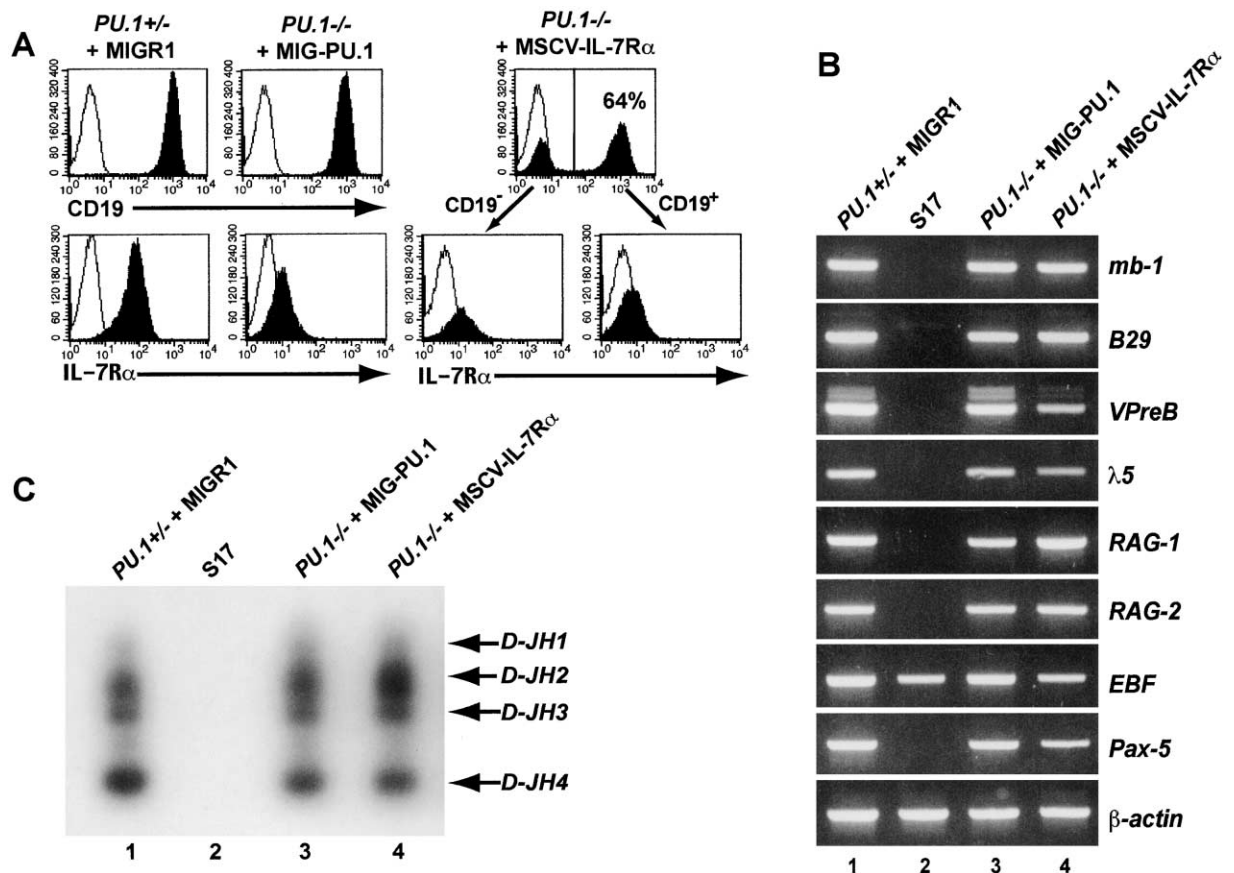


Figure 5. IL-7R $\alpha$ -Transduced, PU.1<sup>-/-</sup> Progenitors Differentiate into Pro-B Cells

(A) Flow cytometric analysis of CD19 and IL-7R expression on control (PU.1<sup>+/-</sup> + MIGR1), PU.1-rescued (PU.1<sup>-/-</sup> + MIG-PU.1) and IL-7R $\alpha$ -rescued (PU.1<sup>-/-</sup> + MSCV-IL-7R $\alpha$ ) cell lines. IL-7R expression on IL-7R $\alpha$ -rescued cells is shown after gating on CD19<sup>+</sup> or CD19<sup>-</sup> cells.

(B) RT-PCR analysis of B lineage gene expression. Total RNA was prepared from the cell lines indicated above as well as from the S17 stromal cell line as a negative control. Transcripts for the indicated genes were analyzed as described in the legend to Figure 1. EBF expression in a stromal cell line has been previously noted (Nutt et al., 1997).

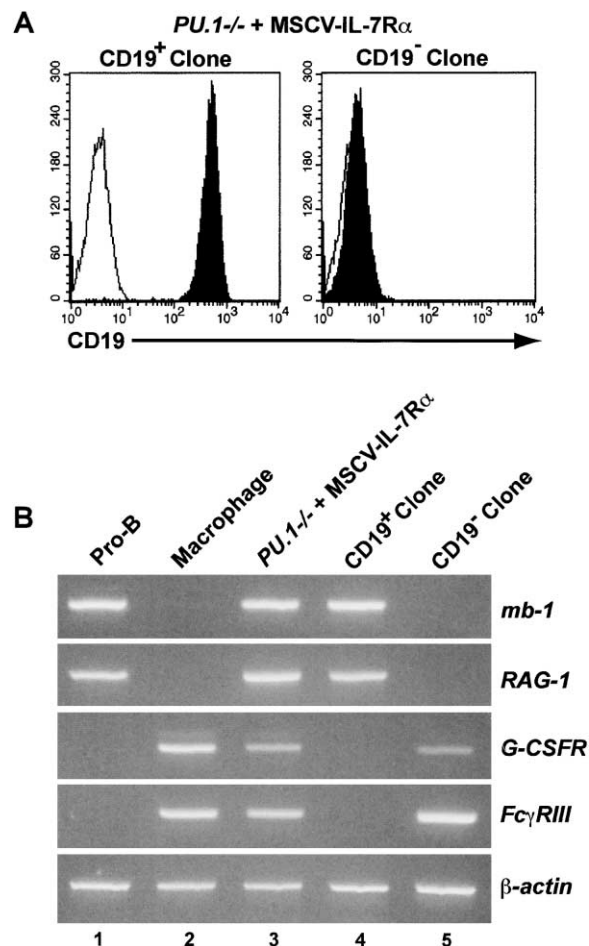
(C) PCR analysis of immunoglobulin heavy-chain (IgH) D-J recombination. Genomic DNA was prepared from the cell lines described above, as well as from the S17 stromal cell line as a negative control. DNA was analyzed by PCR using a primer 5' to most DH segments and a primer 3' of JH4. PCR products were separated using agarose gel electrophoresis and visualized by Southern blotting with a radiolabeled DHFL16-JH4 probe.

mal pattern of B lineage gene expression and IgH DNA rearrangements. Furthermore, we demonstrate that early B lineage differentiation can occur in the absence of the PU.1-related proteins Spi-B or Spi-C. These results suggest that PU.1 is not essential for expression of other B lineage transcription factors, such as E2A, EBF, or Pax5, and may not function in concert with them to regulate transcription of the *mb-1*, *B29*,  $\lambda$ 5, *VpreB*, and *Rag* genes. Instead, PU.1 likely functions in a parallel pathway to regulate early B cell development by controlling the expression of the IL-7R as well as additional genes (see below). Restoration of IL-7R signaling in PU.1<sup>-/-</sup> progenitors can initiate or sustain the B lineage differentiation program, albeit inefficiently. Interestingly, the IL-7R is thought to provide two types of signals in developing lymphoid progenitors, (1) proliferation, and (2) differentiation (reviewed in Hofmeister et al., 1999). These signals can be separated by mutation of specific amino acid residues in the cytoplasmic tail of the receptor (Corcoran et al., 1996). IL-7R signaling appears to

induce IgH locus accessibility and transcription of the *Pax5* gene in pro-B cells (Corcoran et al., 1998). Therefore, loss of IL-7R expression caused by mutation of the *PU.1* gene likely results in defects in both proliferation and differentiation of lymphoid progenitors.

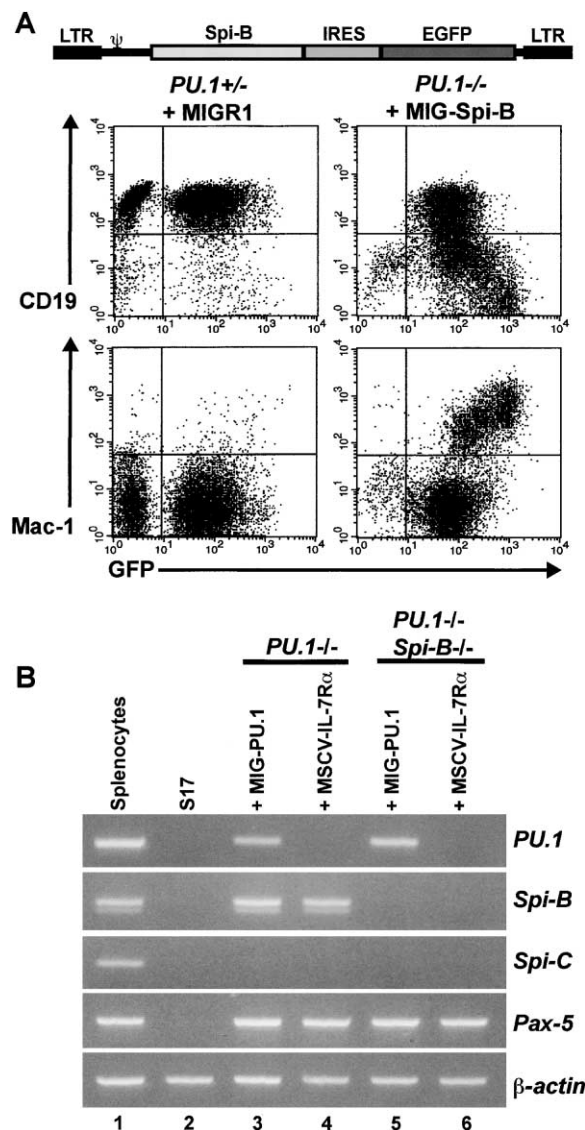
Although expression of IL-7R in PU.1<sup>-/-</sup> progenitors enables early B cell differentiation, significantly fewer pro-B cells are generated in this case than by the restoration of PU.1 expression. This could be due to the following possibilities: (1) PU.1 can rescue more primitive mutant progenitors than the IL-7R; (2) IL-7R promotes survival of mutant progenitors that then undergo stochastic, PU.1-independent differentiation, which is inefficient; or (3) ectopic expression of IL-7R redirects the fate of nonlymphoid mutant progenitors. We note that the defect to B cell development caused by mutation of *PU.1* is more severe than that caused by loss of the IL-7R, since bone marrow and thymus from IL-7R $\alpha$ <sup>-/-</sup> mice does contain low frequencies of B and T cells, respectively (Peschon et al., 1994; Maki et al.,





**Figure 6.** Analysis of *IL-7Rα*-Transduced, *PU.1*<sup>-/-</sup> Clones  
(A) Flow cytometric analysis of CD19. *IL-7Rα*-transduced, *PU.1*<sup>-/-</sup> clones were isolated by plating in methylcellulose. Left and right panels show a CD19<sup>+</sup> and CD19<sup>-</sup> clone, respectively.  
(B) RT-PCR analysis of lymphoid and myeloid gene expression in *IL-7Rα*-transduced, *PU.1*<sup>-/-</sup> clones. RNA was prepared from wild-type pro-B cells, macrophages, or *IL-7Rα*-transduced, *PU.1*<sup>-/-</sup> cells. The CD19<sup>+</sup> and CD19<sup>-</sup> clones (lanes 4 and 5) were isolated from the *IL-7Rα*-transduced, *PU.1*<sup>-/-</sup> cell line (*PU.1*<sup>-/-</sup> + MSCV-IL-7Rα, lane 3). The indicated transcripts were analyzed by RT-PCR after normalization with *β-actin*.

1996). Therefore, mutation of the *PU.1* gene likely results in defects in lymphoid progenitors in addition to loss of *IL-7R* expression. The *PU.1* mutation may also cause arrest of lymphoid development at a stage earlier than that caused by mutation of *IL-7Rα*. We have previously suggested that mutation in *PU.1* causes developmental arrest at the level of a common lymphoid-myeloid progenitor (Singh, 1996; Scott et al., 1997). Interestingly, the *IL-7R* is first expressed on a fetal hematopoietic progenitor with both lymphoid and myeloid potential (Mebius et al., 2001). These cells might be more severely affected in *PU.1*<sup>-/-</sup> than in *IL-7Rα*<sup>-/-</sup> fetuses as a result of defects in expression of additional genes, some of which may encode adhesion receptors such as integrins (Fisher et al., 1999). Thus we favor the possibility that *PU.1* rescues more primitive mutant progenitors to differentiate into pro-B cells by virtue of regulating the



**Figure 7.** *Spi-B* Can Promote Differentiation of *PU.1*<sup>-/-</sup> Progenitors into Pro-B Cells, but Is Not Required  
(A) *Spi-B* rescues *IL-7*-dependent proliferation of *PU.1*<sup>-/-</sup> progenitors and promotes differentiation into pro-B cells as well as macrophages. A schematic structure of the MIG-Spi-B retroviral construct is shown. Lin<sup>-</sup> progenitors (*PU.1*<sup>+/-</sup> or *PU.1*<sup>-/-</sup>) were transduced with MIGR1 or MIG-Spi-B retroviral vectors by coculture with packaging cell lines. Transduced progenitor cells were expanded for 10–14 days on S17 stromal cells with *IL-7*. Cells were analyzed by flow cytometry for expression of CD19 and GFP (upper panels) or Mac-1 and GFP (lower panels).  
(B) RT-PCR analysis of gene expression in *IL-7Rα*-transduced, *PU.1*<sup>-/-</sup> or *PU.1*<sup>-/-</sup> *Spi-B*<sup>-/-</sup> cell lines. Total RNA was prepared from wild-type splenocytes as a positive control, S17 stromal cells as a negative control, or from the indicated *PU.1*<sup>-/-</sup> and *PU.1*<sup>-/-</sup> *Spi-B*<sup>-/-</sup> pro-B cell lines. *PU.1*, *Spi-B*, *Spi-C*, and *Pax5* transcripts were analyzed as described in the legend to Figure 1.

expression of *IL-7R* as well as additional genes that may promote the interaction of lymphoid progenitors with stromal cells.  
*PU.1* is the first transcription factor shown to regulate the *IL-7Rα* gene in lymphoid progenitors. Although the



promoter sequence of the *IL-7R $\alpha$*  gene was reported several years ago, the only transcription factor binding site to be analyzed was an interferon regulatory element capable of binding IRF-1 and IRF-2 (Pleiman et al., 1991). *IRF-2*<sup>-/-</sup> mice have reduced numbers of IL-7-responsive progenitors but it has not been determined if they have impaired expression of the IL-7R (Matsuyama et al., 1993). The site in the *IL-7R $\alpha$*  gene bound by PU.1 in vitro as well as in vivo resides approximately 800 bp downstream of the transcription start site. Intriguingly, this site is embedded in a conserved block of DNA sequence (100 bp) in the murine and human *IL-7R $\alpha$*  genes. Therefore, this region may represent a PU.1-dependent enhancer that regulates expression of the *IL-7R $\alpha$*  gene. It should be noted that the transcription factors E2A, EBF, and Pax5, although required for early B lineage differentiation, do not appear to regulate the expression of the IL-7R (Bain et al., 1994; Lin and Grosschedl, 1995; O'Riordan and Grosschedl, 1999; Nutt et al., 1997). Therefore, control of IL-7R expression in early lymphoid development is a function uniquely provided by PU.1. We note that in later stages of B cell and T cell development, expression of PU.1 no longer correlates with IL-7R $\alpha$  expression. As pro-B cells differentiate into pre-B cells, expression of IL-7R $\alpha$  is downregulated (Sudo et al., 1993) while PU.1 continues to be expressed (our unpublished data). Furthermore, as thymic T cells mature, they shut down expression of PU.1 while IL-7R $\alpha$  continues to be expressed at selected stages of positive selection and then in mature peripheral T cells (Sudo et al., 1993). Therefore other transcription factors must regulate *IL-7R $\alpha$*  gene expression during later stages of B or T cell differentiation.

Retroviral transduction of *PU.1*<sup>-/-</sup> progenitors with *Spi-B* cDNA led to the development of both pro-B cells and macrophages. Therefore *Spi-B* is capable of functionally substituting for PU.1 in B cell as well as macrophage development. Previously, PU.1 and *Spi-B* have been suggested to have redundant functions in regulating B cell activation and terminal differentiation (Garrett-Sinha et al., 1999). Murine PU.1 and *Spi-B* are 67% identical in their Ets domains and have indistinguishable DNA binding specificities (Ray-Gallet et al., 1995; Rao et al., 1999). PU.1 and *Spi-B* are therefore a new example of related transcription factors that serve different functions in vivo as a result of their differing expression patterns. For example, GATA-2 and GATA-3 transcription factors can partially substitute for GATA-1 deficiency when expressed in *GATA-1*<sup>-/-</sup> erythroid cells but do not compensate in vivo because they are not highly expressed in the erythroid lineage (Tsai et al., 1998; Takahashi et al., 2000). It remains to be determined if *Spi-B* expressed under the control of PU.1 regulatory elements can fully complement the PU.1 mutation in vivo.

Taken together with previous findings, the results reported here demonstrate that a major function of PU.1 in hematopoiesis is to regulate expression of myeloid and lymphoid cytokine receptor genes. However, unlike the case with *IL-7R $\alpha$* , transduction of *PU.1*<sup>-/-</sup> progenitors with *c-fms* or *GM-CSFR $\alpha$*  cDNAs restores cytokine-dependent proliferation but not differentiation (DeKoter et al., 1998; Singh et al., 1999). PU.1 controls myeloid development by regulating transcription of multiple genes in addition to those encoding cytokine receptors.

Thus it appears that there are fewer PU.1 target genes in developing B lineage cells compared with macrophages and granulocytes. We have previously demonstrated that higher concentrations of PU.1 protein are required to promote macrophage than B cell development (DeKoter and Singh, 2000). Assuming that the levels of nuclear PU.1 protein are limiting, we speculate that the requirement for a higher concentration threshold of PU.1 protein in macrophage differentiation may reflect the need for effective occupancy of a larger set of genomic targets.

## Experimental Procedures

### Cell Lines

The retroviral packaging cell lines Phoenix (Kinsella and Nolan, 1996) and GP+E-86 (Markowitz et al., 1988), as well as NIH-3T3 cells, were maintained in complete DMEM medium. S17 stromal cells (Collins and Dorshkind, 1987), J558-IL-7 cells (Winkler et al., 1995), and 38B9 pro-B cells (Alt et al., 1981) were maintained in complete RPMI-1640 medium. All complete media contained 10% fetal bovine serum (Hyclone), 100 U/ml penicillin/streptomycin, 2 mM L-glutamine, 5 × 10<sup>-5</sup> M  $\beta$ -mercaptoethanol, and 0.5 mM HEPES.

### Construction of Retroviral Vectors and Packaging Cell Lines

The murine stem cell virus-internal ribosomal entry site-green fluorescent protein (MSCV-IRES-GFP, MIGR1) retroviral vector has been described (Pear et al., 1998). MIG-PU.1 was generated by blunt-end ligation of HA-tagged *PU.1* cDNA into the HpaI site of MIGR1. MSCV was generated by removal of the PGK-neo cassette from MSCV-neo vector (Hawley et al., 1994) by digestion with BglII and BamHI. The ends were blunted and the plasmid was recircularized using T4 DNA ligase. MSCV-IL-7R $\alpha$  was generated by blunt-end ligation of *IL-7R $\alpha$*  cDNA (Lo et al., 1999) into the HpaI site of MSCV. Murine *Spi-B* cDNA was amplified by RT-PCR using RNA prepared from IL-7-dependent pro-B cell lines, high-fidelity TAQ polymerase (Takara), and primers complementary to the 5' and 3' ends of the cDNA (see Supplemental Table S1 at <http://www.immunity.com/cgi/content/full/16/2/297/DC1>). These primers incorporated NotI and ApaI restriction sites into the cDNA for cloning into pcDNA3-HA (Brass et al., 1996). HA-tagged *Spi-B* cDNA was cut from pcDNA3-HA-*Spi-B* by HindIII and ApaI digestion and subcloned into the HpaI site of MIGR1 by blunt-end ligation.

To establish retroviral packaging cell lines, retrovirus produced by transient transfection of Phoenix cells was used to infect GP+E-86 cells after overnight treatment with tunicamycin. After 2 days, the brightest GFP-expressing cells were sorted using a Becton-Dickinson FACScalibur system and expanded under *gpt* selection. Sorted cell lines were analyzed periodically to ensure they consisted of GFP<sup>+</sup> cells. The retroviral titer of each cell line was determined by infection of NIH-3T3 cells followed by flow cytometric analysis of GFP. Expression of HA-tagged PU.1 or *Spi-B* in the MIG-PU.1 and MIG-*Spi-B* packaging cell lines was confirmed by Western blotting using anti-PU.1 and/or anti-HA antibodies. Expression of IL-7R $\alpha$  by the MSCV-IL-7R $\alpha$  packaging cell line was confirmed by flow cytometry.

### Isolation of Lineage-Depleted Fetal Hematopoietic Progenitors

*PU.1*<sup>+/-</sup> or *PU.1*<sup>+/-</sup> *Spi-B*<sup>-/-</sup> mice were mated to produce *PU.1*<sup>-/-</sup> and *PU.1*<sup>-/-</sup> *Spi-B*<sup>-/-</sup> fetuses as well as control littermates. The presence of a vaginal plug on the morning after matings was taken as 0.5 d.p.c. Fetuses (14.5 d.p.c.) were phenotyped using flow cytometric analysis of Mac-1 expression (Scott et al., 1997). Genotyping of *PU.1* alleles was performed by PCR using a combination of three primers recognizing *PU.1* exon 4, *PU.1* exon 5, and the PGK promoter (Supplemental Table S1 at <http://www.immunity.com/cgi/content/full/16/2/297/DC1>). Genotyping of *Spi-B* alleles was performed using a combination of three primers recognizing *Spi-B* exon 5, *Spi-B* exon 6, and the PGK promoter (Supplemental Table S1 at <http://www.immunity.com/cgi/content/full/16/2/297/DC1>). Lineage depleted (Lin<sup>-</sup>) hematopoietic progenitors were isolated from 14.5 d.p.c. fetal liver suspensions as described previously (DeKoter and Singh, 2000).

### Infection and Culture of Lineage-Depleted Fetal Hematopoietic Progenitors

GP+E-86 lines were irradiated using 2000 rad from a Cesium source and plated at  $1.5 \times 10^5$  cells/well in a 24-well plate (Corning-Costar) and incubated overnight. Lin<sup>-</sup> fetal liver progenitors ( $10^5$ ) were introduced in each well and infected by coculture for 2 days in complete IMDM medium containing 4  $\mu$ g/ml polybrene and 5% conditioned medium from J558-IL-7 cells. Infected cells were removed by gentle pipetting and transferred onto a monolayer of irradiated S17 stromal cells. Cultures were fed with fresh IL-7-containing medium every 4 days and analyzed after 10–14 days. All experiments were performed at least three times unless otherwise indicated. The statistics shown represent means and standard error of *n* experiments.

### Establishment of IL-7-Dependent Cell Lines and Clones

Cells from 10–14 day cultures of *PU.1*<sup>+/-</sup> and *PU.1*<sup>-/-</sup>, or *PU.1*<sup>-/-</sup> *Spi-B*<sup>-/-</sup> fetal hematopoietic progenitors rescued with *PU.1*, *Spi-B*, or *IL-7R $\alpha$*  cDNAs were expanded and passaged by plating on monolayers of irradiated S17 stromal cells and IL-7-containing medium as described above. IL-7-dependent cell lines were passaged by plating at  $1.5 \times 10^5$  cells/ml every 4 days. To generate pro-B cell clones, early-passage IL-7-dependent cell lines were plated at low density (up to 100 cells) in 35 mm tissue culture dishes with irradiated S17 stromal cells and IL-7-containing methylcellulose medium (StemCell Technologies). After 7–10 days, well-separated colonies were picked and propagated as described above.

### Antibodies and Flow Cytometry

Flow cytometric analyses were performed on single-cell suspensions washed in Dulbecco's phosphate-buffered saline containing 0.05 M EDTA and 0.5% LPS-free bovine serum albumin (Sigma). Cells were stained with phycoerythrin (PE) or biotin-conjugated antibodies and streptavidin(SA)-PE, SA-allophycocyanin (APC), or PE-conjugated anti-rat secondary reagents. Propidium iodide (Molecular Probes) was used to exclude dead cells from analysis. Anti-mouse monoclonal antibodies used in this study were purchased from Pharmingen unless otherwise indicated and used according to the manufacturer's instructions. The antibodies used included RA3-6B2-biotin (B220, CD45RA); 145-2C11-biotin (CD3e); H129.19-biotin (CD4); 53-7.3-biotin (CD5); 53-6.7-biotin (CD8a); M1/70-PE (CD11b, Mac-1); Ter119-biotin; RBC-8C5-biotin (Gr-1); 1D3-PE (CD19); VP245 (rat anti-VpreB [Karasuyama et al., 1993], gift of Y. Argon); M1/69-PE (CD24, HSA); S7-PE (CD43, leukosialin); B12-1-biotin (CD127, IL-7R $\alpha$ ); and II/41-biotin (Ig $\mu$ ).

### RT-PCR Analysis

RNA was isolated using RNAzol (Tel-Test) and reverse-transcribed using a first-strand cDNA synthesis kit (Pharmacia-Amersham). RNA concentration was normalized between samples by performing 20 or 25 cycles of PCR on serial dilutions of cDNA using primers for  $\beta$ -actin. PCR was performed for 30 cycles using primers and conditions described in Supplemental Table S1 at <http://www.immunity.com/cgi/content/full/16/2/297/DC1>. PCR products were visualized by ethidium bromide staining and digital photography (Kodak DC120). Each RT-PCR analysis was performed with at least three independent RNA samples.

### PCR Analysis of DH-JH Rearrangement

Genomic DNA was prepared from IL-7-dependent pro-B cell lines using a genomic DNA isolation kit (Promega). *DH-JH* rearranged *IgH* genes were amplified using 700 ng DNA per reaction and 30 cycles. The primers used were complementary to the 5' ends of most DH segments and to the 3' end of the *JH4* segment (Ehlich et al., 1994) (see Supplemental Table S1 at <http://www.immunity.com/cgi/content/full/16/2/297/DC1>). An amplified DHFL16-JH4 fragment was subcloned into pcr-II-TOPO (Invitrogen) and used as a probe for Southern blotting of amplified rearrangements. <sup>32</sup>P-dCTP-labeled probes were generated using a random priming labeling kit (Roche).

### Gel-Shift Assays

PU.1 protein was generated by in vitro translation using 1  $\mu$ g of pcDNA3-HA-PU.1 and a TNT coupled transcription/translation kit (Promega). DNA binding by PU.1 was analyzed using a gel-shift

assay with double-stranded oligonucleotide probes containing the PU.1 binding sites  $\lambda$ B (Brass et al., 1996), IL-7R $\alpha$  site 1, or IL-7R $\alpha$  site 2 (see Supplemental Table S1 at <http://www.immunity.com/cgi/content/full/16/2/297/DC1>). Synthetic complementary oligonucleotides were annealed and end-labeled with  $\alpha$ -<sup>32</sup>P dCTP using the Klenow fragment. DNA binding reactions were performed for 30 min at 25°C and contained  $5 \times 10^5$  cpm of probe, 2 or 4  $\mu$ l of IVT PU.1 protein, and 0.5  $\mu$ g of poly-(dI-dC) in a final volume of 20  $\mu$ l. The resulting complexes were resolved on a 5% native polyacrylamide gel using 1 $\times$  TGE buffer, at 150V for 2 hr.

### Transient Transfection Analysis

pGL3 (Promega) derivatives were constructed so as to have a 3-fold direct repeat of wild-type (5'-AAACAGGAAGTCTG-3') or mutant (5'-AAACAGGACGTCTG-3') PU.1 site 2, upstream of the SV40 promoter. 38B9 pro-B cells ( $5 \times 10^6$ ) were transfected with 10  $\mu$ g of the various reporter constructs and 1  $\mu$ g pRL-TK (Promega) as an internal control. Transfections were performed by electroporation using a Bio-Rad Gene Pulser in 1 ml cuvettes at 25  $\mu$ F and 1500V settings. The cells were lysed 24 hr after transfection and analyzed for both firefly and *Renilla* luciferase activities using a dual luciferase assay kit (Promega) and an Analytical Luminescence Laboratory Monolight 1500 tube luminometer. Transfections were done in duplicate at least three independent times.

### Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation assays were performed as described (Boyd et al., 1998) with a few modifications. Cells ( $5\text{--}10 \times 10^7$ ) were crosslinked for 10 min at room temperature by adding paraformaldehyde (1% final) with gentle rocking. The crosslinking reaction was stopped by the addition of glycine to a final concentration of 0.125 M. Cells were washed in cold PBS and nuclei were isolated with cell lysis buffer (5 mM Pipes [pH 8.0], 85 mM KCl, and 0.5% NP40). Nuclei were resuspended in lysis buffer (50 mM Tris [pH 8.0], 10 mM EDTA, and 1% SDS) and sonicated using a Branson 250 sonifier for two 30 s pulses at constant power and an output setting of 5 to generate a mean DNA size of 0.2–1.0 kb. Chromatin was diluted 1 to 3 in dilution buffer (0.01% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, and 20 mM Tris [pH 8.0]), precleared with Staph A cells (Roche) for 15 min at 4°C. Chromatin from  $5 \times 10^6$  cells was incubated with 1  $\mu$ g of control rabbit IgG (Santa Cruz) or affinity purified anti-PU.1 antibodies for 3 hr, followed by addition of Staph A cells for 15 min at room temperature. The immunoprecipitates were collected by centrifugation and subjected to six rounds of washes. DNA was purified from the input and the immunoprecipitates by phenol/chloroform extraction and ethanol precipitation after reversal of crosslinking by heating at 67°C for 5 hr and a proteinase K digestion, 45°C for 2 hr. Input and immunoprecipitated DNA samples were dissolved in 100  $\mu$ l TE and 5  $\mu$ l of each was analyzed by semiquantitative PCR (30–35 cycles) with primer pairs derived from the *IL-7R $\alpha$* , *c-fms*, *Ig $\kappa$* , and *hprt* genes (see Supplemental Table S1 at <http://www.immunity.com/cgi/content/full/16/2/297/DC1>). For quantitation, 0.1  $\mu$ l of  $\alpha$ -<sup>32</sup>P dCTP (6000 Ci/mmol) was added to the PCR reactions and the PCR products were visualized by autoradiography and analyzed using a PhosphorImager (Molecular Dynamics) after being resolved on a 6% polyacrylamide gel.

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